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Genomics Proteomics Bioinformatics 10 (2012) 302–309

GENOMICS PROTEOMICS & BIOINFORMATICS

www.elsevier.com/locate/gpb

Original Research

MicroRNA Regulation in Extreme Environments: Differential Expression of MicroRNAs in the Intertidal Snail *Littorina littorea* During Extended Periods of Freezing and Anoxia

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Received 26 July 2012; revised 11 September 2012; accepted 12 September 2012
Available online 8 October 2012

Abstract

Several recent studies of vertebrate adaptation to environmental stress have suggested roles for microRNAs (miRNAs) in regulating global suppression of protein synthesis and/or restructuring protein expression patterns. The present study is the first to characterize stress-responsive alterations in the expression of miRNAs during natural freezing or anoxia exposures in an invertebrate species, the intertidal gastropod *Littorina littorea*. These snails are exposed to anoxia and freezing conditions as their environment constantly fluctuates on both a tidal and seasonal basis. The expression of selected miRNAs that are known to influence the cell cycle, cellular signaling pathways, carbohydrate metabolism and apoptosis was evaluated using RT-PCR. Compared to controls, significant changes in expression were observed for miR-1a-1, miR-34a and miR-29b in hepatopancreas and for miR-1a-1, miR-34a, miR-133a, miR-125b, miR-29b and miR-2a in foot muscle after freezing exposure at −6 °C for 24 h ($P < 0.05$). In addition, in response to anoxia stress for 24 h, significant changes in expression were also observed for miR-1a-1, miR-210 and miR-29b in hepatopancreas and for miR-1a-1, miR-34a, miR-133a, miR-29b and miR-2a in foot muscle ($P < 0.05$). Moreover, protein expression of Dicer, an enzyme responsible for mature microRNA processing, was increased in foot muscle during freezing and anoxia and in hepatopancreas during freezing. Alterations in expression of these miRNAs in *L. littorea* tissues may contribute to organismal survival under freezing and anoxia.

Keywords: Freeze tolerance; Anaerobiosis; Metabolic rate depression; Periwinkle; Marine mollusk

Introduction

If humans experience either anoxia (complete depletion of dissolved oxygen) or tissue freezing, they suffer extensive cellular damage, most often leading to death. However, there are organisms that are able to survive such extreme conditions. The common periwinkle, *Littorina littorea*, is a marine snail that inhabits the intertidal zone along the northern Atlantic shores of North America and Europe. Life in the intertide is harsh as conditions in the local microenvironment change constantly with the ebb and flow of the tides daily as well as seasonally [1]. When the tide recedes, gill-breathing snails are often left out of water and are therefore unable to continue aerobic metabolism during aerial exposure [1]. When this happens in winter, snails are also exposed to subzero temperatures, triggering whole body freezing of snails when air temperatures drop below the −1.9 °C freezing point of their body fluids [2]. Periwinkles are remarkably resistant to both of these stresses and can endure continuous anoxia or freezing exposures for several days. Multiple studies have revealed that the regulation of key survival pathways is involved in the tolerance of both of these stresses in *L. littorea* [1–4]. These studies have characterized energy metabolism, enzymatic regulation and alterations in expression of multiple genes and proteins that are involved in anoxia and freezing survival in *L. littorea*.

MicroRNAs (miRNAs) have been recognized as key players in posttranscriptional regulation of protein expres-

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http://dx.doi.org/10.1016/j.gpb.2012.09.002

sion but the involvement of this mechanism in animal adaptation to environmental stress is only now beginning to be analyzed. Marine snails are an excellent model system for studies of resistance to natural multi-stresses besides anoxia/hypoxia and freezing, since the intertidal environment can also expose organisms to conditions of hyperoxia, hyper- or hypo-osmotic stress, heavy metals and pollutants [1]. The present study uses the *L. littorea* model to assess involvement of miRNAs in organismal responses to anoxia and freezing stresses. Tissues selected for examination in the study included hepatopancreas, a liver-like organ that is critical in supporting the anaerobic metabolism and in supplying carbohydrate for fermentation and foot muscle which functions in the attachment of the animal to surrounding rock. Both tissues have been previously shown to adapt during periods of environmental stress [2].

miRNAs are short, non-coding RNA molecules that are capable of regulating protein expression within cells. These non-coding RNAs are transcribed as long primary sequences that are then processed into pre-miRNA by the nuclear enzyme, drosha [5]. Once processed, pre-miRNAs are exported from the nucleus and further processed into mature miRNAs (17–22 nt) by Dicer, a type III RNase enzyme [5]. Mature miRNA transcripts are able to bind with either partial or full complementarity to the 3′ UTR of mRNA targets, leading to either inhibition of mRNA translation or degradation of the mRNA target [6–8]. A single miRNA may target multiple mRNAs, and a single mRNA may have binding sites for multiple miRNAs [9,10]. As a result, this creates a complex regulatory system that has been predicted to be involved in almost every aspect of biological function throughout the animal kingdom. In this regard, miRNAs are known to be critically involved in biological development, cell differentiation, apoptosis, cell cycle control, stress response and disease pathogenesis [6,11–13].

Based on their recently established involvement in responses to environmental stresses in selected vertebrate species (*e.g.*, freeze tolerance in wood frogs, anoxia tolerance in turtles, and hibernation in bats and ground squirrels [14–17]), we reasoned that miRNAs are likely to play a role in anoxia and freeze tolerance in *L. littorea*. Changes in miRNA expression may be crucial to transducing stress signals into appropriate changes in mRNA translation to support hypoxia/anoxia survival or aid cryoprotection. Since there is no genomic information available for the common periwinkle, in the present study, the expression of only highly-conserved miRNAs with previously established roles in metabolic rate depression in other organisms were selected for evaluation.

Results

We used a modified stem-loop RT-PCR to amplify the selected miRNAs that are highly-conserved across different species [18]. The miRNAs include miR-1a-1, miR-210, miR-34a, miR-133a, miR-125b, miR-29b and miR-2a. Consensus sequences for these miRNAs were taken from other invertebrate sources, including *Caenorhabditis elegans* (cel), *Drosophila melanogaster* (dme), *Daphnia pulex* (dpu) and *Lottia gigantea* (lgi). These miRNAs were amplified from total RNA from foot muscle and hepatopancreas of *L. littorea*.

All miRNA PCR products were confirmed by sequencing. Sequences were aligned with those available from miRBase (http://www.mirbase.org/) and showed a high degree of conservation with their counterparts (**Figure 1**). It is noted that all miRNAs contained identical nucleotides at positions 2–8 with their counterparts. This region is known as the "seed" region, which is typically the mRNA-binding region of the miRNA [19], suggesting that they possibly have similar mRNA targets.

Expression of miRNAs in foot muscle

Expression of seven miRNAs was analysed by RT-PCR in foot muscle from control snails (10 °C acclimated, representing an anoxic condition) and snails exposed to freezing at −6 °C for 24 h (freezing) or to a nitrogen gas atmosphere at 10 °C for 24 h (anoxia). As shown in **Figure 2**, compared to controls, significant increases in the expression of most of the miRNAs examined were detected in freezing-exposed snails ($P < 0.05$). For example, expression of miR-1a-1 was increased more than 2.5-fold (2.65 ± 0.11). However, no significant alteration in the expression of miR-210 was noticed in response to freezing. In addition, a similar trend was observed for the miRNA expression in response to anoxia. Expression of five miRNAs increased significantly, including miR-1a-1, miR-34a, miR-133a, miR-29b and miR-2a, compared to controls ($P < 0.05$) (Figure 2). However, expression of miR-215

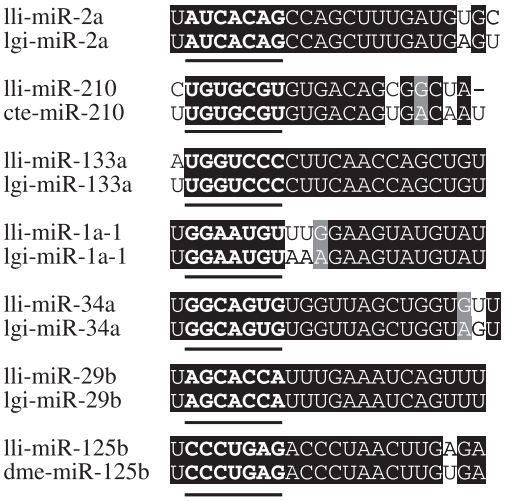

Figure 1 Conserved miRNAs amplified from *L. littorea*
miRNAs were amplified using stem-loop RT-PCR. The sequences were aligned with their respective counterparts from other invertebrate species. Lgi: owl limpet *Lottia gigantean*; dme: fruit fly *Drosophila melanogaster*; cte: polychaete worm *Capitella teleta*. The seed sequences were indicated in bold and underlined and the conserved nucleotides were highlighted in black background.

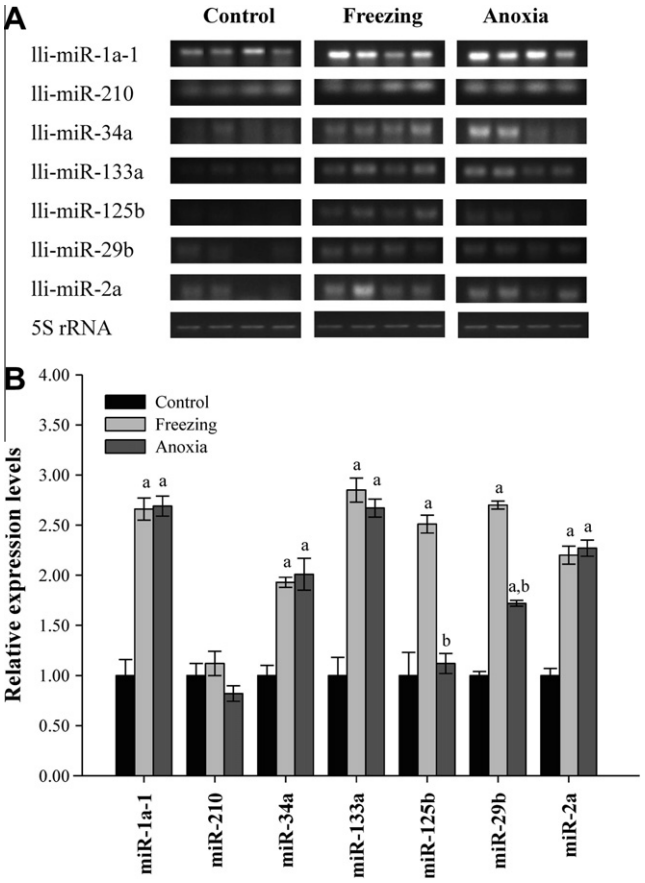

Figure 2 Effect of freezing or anoxia on miRNA expression in the foot muscle of *L. littorea*

A. Representative RT-PCR data for miRNAs in the foot muscle from control snails and snails exposed to freezing and anoxia for 24 h, respectively. **B.** Relative expression of miRNAs examined in panel A. Histogram shows expression levels of different miRNAs after normalized with that of 5S rRNA. Data were presented as mean ± SEM, for 3–4 independent samples from separate animals. a indicates significant difference from the corresponding control ($P < 0.05$) and b indicates significant difference from the freezing group ($P < 0.05$).

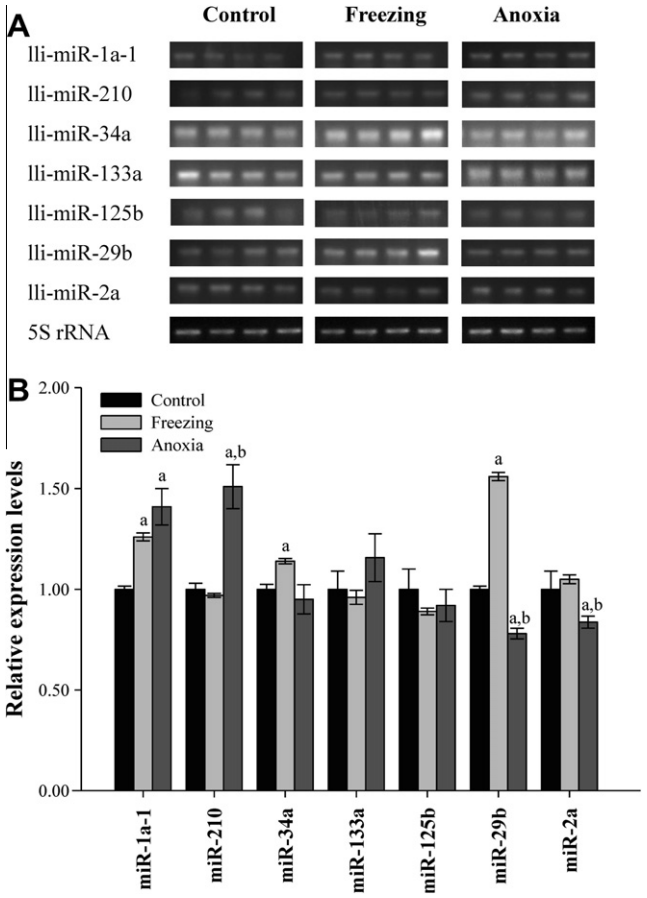

Figure 3 Effect of freezing or anoxia on miRNA expression in the hepatopancreas of *L. littorea*

A. Representative RT-PCR data for miRNAs in the hepatopancreas from control snails and snails exposed to freezing and anoxia for 24 h, respectively. **B.** Relative expression of miRNAs examined in panel A. Histogram shows expression levels of different miRNAs after normalized with that of 5S rRNA. Data were presented as mean ± SEM, for 3–4 independent samples from separate animals. a indicates significant difference from the corresponding control ($P < 0.05$) and b indicates significant difference from the freezing group ($P < 0.05$).

didn't change in response to anoxia, which is significantly different from the expression under freezing exposure. Moreover, although the expression of miR-29b was increased significantly in response to anoxia compared to control, the augmentation was significantly low, when compared to those exposed to freezing for the same period of time. No significant changes in expression were found for miR-210 during anoxia.

Expression of miRNAs in hepatopancreas

Expression of seven aforementioned miRNAs were similarly analysed in hepatopancreas tissue from control snails and snails exposed to freezing or to anoxia. As shown in **Figure 3**, compared to controls, significant increases in the expression of three miRNAs (miR-1a-1, miR-34a and miR-29b) were detected in freezing-exposed snails ($P < 0.05$), although the increases were <2-folds in all cases. The expression of the remaining four miRNAs was comparable in freezing-exposed snails to that in control snails. However, in response to anoxia, hepatopancreas showed significant increases in expression of miR-1a-1 and miR-210, whereas a significantly – reduced expression was detected for miR-29b and miR-2a ($P < 0.05$) (Figure 3). Levels of the other three miRNAs didn't change significantly under anoxia.

Protein expression of Dicer

Dicer is a crucial protein in processing of precursors into mature miRNA. We therefore went forward to examine its relative expression at the protein level. The immunoblotting analysis indicated that relative expression of Dicer protein in foot muscle increased by 2.51 and 3.13-fold after exposed to freezing and anoxia, respectively ($P < 0.05$) (**Figure 4**). Significantly increased expression of Dicer was also

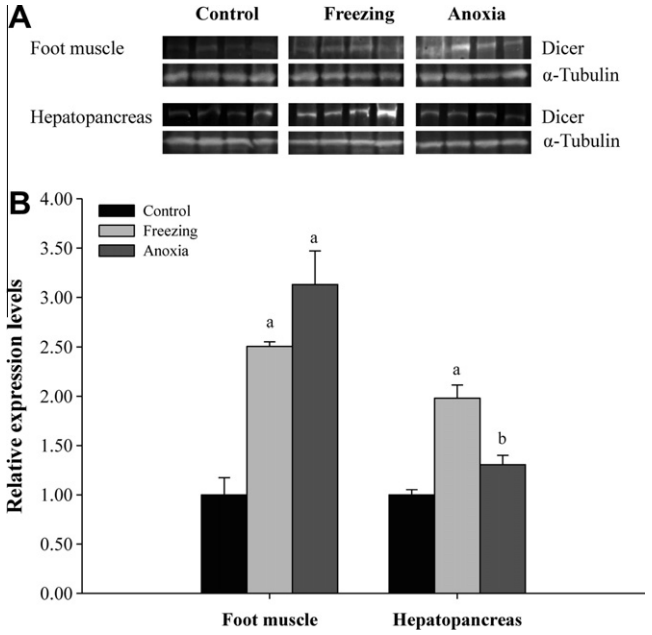

Figure 4 Effect of freezing and anoxia on protein expression of Dicer in *L. littorea*

A. Representative immunoreactive data for Dicer in foot muscle and hepatopancreas from control snails and snails exposed to freezing and anoxia for 24 h, respectively. **B.** Quantification of relative expression of Dicer protein shown in panel A. Histogram shows protein expression levels of Dicer after normalized with that of α tubulin. Data were presented as mean ± SEM, for 3–4 independent samples from separate animals. a indicates significant difference from the corresponding control ($P < 0.05$) and b indicates significant difference from the freezing group ($P < 0.05$).

detected in hepatopancreas from snails subjected to freezing treatment (1.98-fold). However, for the snails exposed to anoxia, a modest but insignificant increase in Dicer expression was observed in hepatopancreas (Figure 4).

Discussion

Previous research has indicated that both anoxia and freeze tolerance are linked and that many similar biochemical mechanisms support survival in both states [2]. This finding is logical since the freezing of snail hemolymph effectively halts oxygen transport to tissues, thereby imposing hypoxia and ultimately full anoxia on tissues. During periods of severe environmental stress such as freezing and anoxia, tolerant animals generally respond with a suppression of energy-expensive cell functions including transcription and translation [1]. Only about 1% of mRNA transcripts show an overall decrease in expression during anoxia-induced metabolic rate depression in *L. littorea* but there is an overall decrease in protein synthesis of about 50% [1]. Considering the role that miRNAs often play in the inhibition of mRNA translation, an increase in miRNA expression could be one way to establish a general reduction of protein synthesis without decreasing overall mRNA levels. Indeed, many cellular functions have been experimentally shown to be regulated by miRNAs and several algorithm-based databases have been developed to predict miRNA targets in both sequenced vertebrate and invertebrate species [7,16]. In this study, the potential roles for all miRNAs investigated were inferred from known and putative targets generalized from other species.

Mature miRNAs are excised from pre-miRNA transcripts by the type III RNase enzyme, Dicer. Changes in the amount or activity of the Dicer enzyme when snails freeze or are anoxic could influence the levels of total mature miRNA that are processed and thereby alter the translational state of the mRNA transcripts under their control. The present study found that Dicer protein increased significantly in the foot muscle of frozen and anoxic snails but only increased during freezing in hepatopancreas. Interestingly, although the expression of individual mature miRNA is controlled by the combined actions of transcriptional factors and RNA processing enzymes, such as Dicer, the expression pattern of Dicer protein does approximately match the overall expression patterns of miRNA. These results suggest that enhanced Dicer levels may elevate mature miRNA processing activity in the stressed state, thereby contributing to the significant increase in miRNA levels that, in turn, influence a reduction in net protein synthesis.

Similar to our findings, a recent study identified miR-210 as hypoxia-inducible in human breast cancer (MCF7 and MDA-MB231) cell lines [20,21]. The expression of miR-210 is thought to be regulated by the hypoxia-inducible transcription factor HIF-1α [22], a transcription factor previously shown to be upregulated during anoxia in *L. littorea* [1]. In vertebrate studies miR-210 has been shown to repress mitochondrial respiration by targeting transcripts coding for iron-sulfur cluster assembly (*ISCU1/2*) proteins involved in electron transport function and mitochondrial reduction–oxidation reactions [23,24]. Interestingly, miR-210 showed a significant increase in response to anoxia in hepatopancreas of *L. littorea*. Consequently, miR-210 may act to repress mitochondrial respiration and associated downstream functions in response to anoxia in hepatopancreas tissue. This makes sense as hepatopancreas has been previously shown to undergo an anoxia-induced metabolic shift to primarily glycolysis from oxidative phosphorylation [3], a process that may include regulation by miR-210.

In hypoxia studies, similar to those discussed above, a consistent decrease in miR-29b has also been observed in human breast cancer cell lines [20]. This is in contrast with the increase in miR-29b levels observed in foot muscle from anoxic and frozen snails and from hepatopancreas of frozen *L. littorea.* Interestingly, miR-29b showed a significant decrease in anoxic hepatopancreas. The miR-29b miRNA is predicted to target phosphoinositide 3-kinase (PI3K), a member of the PI3K/Akt pathway [25]. A general increase in miR-29b levels in *L. littorea* tissues during freezing hepatopancreas could contribute to a survival strategy involving a reduction in protein synthesis [26]. Previous experiments using *L. littorea* showed that protein translation was strongly suppressed by 50% within just 30 min of anoxic

exposure and was maintained at low levels over 48 h of anoxia exposure; however, this study did not explore the possibility of freezing-induced suppression of translation [27]. Interestingly, a strong regulator of protein translation is the PI3K/Akt pathway through the regulation of mTOR, a process that could be negatively controlled by an increase of miR-29b [28]. In addition, previous studies have shown that targeting of *PI3K* by the miR-29 family indirectly enhances p53 activity [29]. This finding could indicate a role for p53 induced cell cycle arrest and genomic stability during freezing in *L. littorea*. Similar to the expression of miR-29b, miR-34a also showed a significant increase in expression in anoxic and frozen foot muscle and frozen hepatopancreas. This miRNA has been identified as a pro-cell cycle arrest factor that is also a direct transcriptional target of p53 [30]. When taken in context with the observed changes in expression levels in miR-29b, an increase in miR-34a further strengthens the possible involvement of p53 in a tissue and stress-specific manner. As p53 has an established role in promoting anaerobic metabolism during periods of oxidative stress [31], it makes sense that both miR-29b and miR-34a are suppressed in anoxic hepatopancreas; perhaps allowing the continued function of p53 and promoting a metabolic shift to glycolysis [3].

Expression of miR-125b, the homologue of the *C. elegans* miRNA lin-4, was freezing-responsive in *L. littorea* foot muscle. This miRNA is known to be cold-inducible in zebrafish embryos and is predicted to play a critical role in preventing the translation of proteins involved in apoptosis, cell cycle arrest and fine-tuning the p53 network activity [31–32]. It has been shown in vertebrate models that miR-125b is able to regulate the p53 pathway by directly targeting the p53 transcript [33–34]. Apart from possible roles in anti-apoptosis and p53 regulation, miR-125b has also been shown to activate the NF-κB pathway, a pathway known to increase cellular antioxidant defense mechanisms, decrease apoptosis, and increase transcription of p53 and miR-34a. miRNA-125b is able to exert control over NF-κB signaling through the regulation of tumor necrosis factor, alpha-induced protein 3 (*TNFAIP3*), a negative NF-κB regulator [35–36]. Activation of the NF-κB pathway could act to increase antioxidant defense mechanisms during periods of low oxygen stress, perhaps in preparation for dealing with a rapid increase in oxidative stress when oxygen is reintroduced into tissues [37]. Interestingly, previous research in *L. littorea* has shown that the gene expression of key components involved in oxidative defense increases during both freezing and anoxia [1–4]. Inhibition of apoptosis is an important component of long-term metabolic rate depression, a state in which cellular replacement processes must be minimized to facilitate long-term survival [38]. Interestingly, an anti-apoptotic state may also be established in foot muscle by miR-2a, a primarily invertebrate miRNA, by targeting the pro-apoptotic protein, *Reaper* [39]. The miRNA-2 family is the largest miRNA group in many invertebrates and was one of the first discovered in *D. melanogaster*. Given the tissue specific expression of miR-2a, *L. littorea* foot muscle may be more resistant to apoptosis. As the foot muscle is critical in the attachment of the animal to surrounding rock, atrophy of this tissue may be detrimental to survival.

Similar changes in expression of muscle-specific miRNAs, miR-1a-1 and miR-133a, were detected in response to both 24 h freezing and anoxia in foot muscle. Parallel expression patterns of these miRNAs were expected in foot muscle tissue since both miRNAs are typically involved in myocyte proliferation and differentiation [40]. miRNA-1a-1 has been shown to positively regulate the expression of *Mef2a* and *Gata4*, which are key transcription factors that promote muscle maintenance during periods of environmental stress [41]. Similar increases were observed in miR-133a under both freezing and anoxic conditions in foot muscle.

Previous research has shown that *L. littorea* displays a selective upregulation of genes that are necessary for stress survival [1–4]. The expression patterns and proposed targets of miRNA examined in this study suggest that these small non-coding RNA may support pro-survival responses including translational and cell cycle arrest, increased antioxidant defense and decreased anti-apoptosis. In conclusion, it seems very clear from studies in vertebrates and now this study in invertebrates that miRNAs may play roles in stress tolerance. This work represents an initial foray into the importance of miRNAs in anoxia and freezing tolerance in an invertebrate species, and also provides a primary annotation of miRNAs in the common periwinkle, *L. littorea.*

Materials and methods

Animals and treatments

Collection and treatment of snails was similar to that described in English and Storey [2]. Briefly, common periwinkles (*L. littorina*) were washed in tap water and then rinsed in sea water. They were placed in covered tanks of aerated full strength sea water (34.74 g/L Instant Ocean Sea Salt in distilled water) at ~10 °C in an incubator for 1 week prior to use. Snails were not fed but seawater was changed periodically. For freezing experiments, nucleation temperature ranged from −6.7 to −7.5 °C over the first hour, and the incubator was then set to −6.0 °C for the remainder of the 24 h of freezing exposure. For anoxia exposure, snails were placed in jars with ~1 cm of seawater at the bottom; the jars had been previously flushed with nitrogen gas (bubbler inserted in the water) for 30 min via a gas line attached to a port in the lid. After adding the snails the water was again bubbled with nitrogen gas for a further 15 min and then the gas lines were removed and the ports sealed. Jars were held at 10 °C over a 24 h anoxia exposure and then the gas lines were reconnected during sampling. All snails were sampled by quickly breaking the shell and then rapidly dissecting out foot muscle

and hepatopancreas; tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Protein isolation

Soluble proteins were extracted from frozen tissue of individual snails using homogenizing buffer (20 mM Hepes pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate) containing PMSF and protease inhibitor cocktail (Sigma) as described previously [17]. Protein concentrations were assessed using the BioRad protein assay (Cat# 500-0006). The samples were denatured by mixing with equal volume of 2× SDS loading buffer (100 mM Tris-base, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercaptoethanol) and incubating in boiling water for 5 min. Samples were stored at −80 °C until use.

Immunoblotting

Aliquots containing 20 μg protein were separated with 8% polyacrylamide gels using a discontinuous buffer system and then transferred onto polyvinylidenedifluoride (PVDF) membrane (Cat# IPVH00010, Millipore) using a BioRad mini Trans-Blot cell. Immunblotting was performed as described previously [17]. After blocking, the membranes were incubated with rabbit polyclonal anti-Dicer primary antibody (1:1000 v:v dilution, Cat# sc-30226, Santa Cruz) for 24 h at 4 °C and then with anti-rabbit HRP-conjugated secondary antibody (1:1000 v:v dilution; Cell Signaling, Cat# 7074) for 1 h at 21 °C after wash. The immunoreactive signals were then detected using enhanced chemiluminescence and band densities were quantified. Subsequently, gels were stained with Coomassie blue and used to normalize protein loading.

RNA isolation

Total RNAs were extracted from frozen foot muscle or hepatopancreas tissue from individual animals using Trizol® reagent (Invitrogen) as described previously [17]. The quality of all RNA isolations were validated with an OD_{260}/OD_{280} ratio of 1.8–2.0. Further quality validation was done by running 2 μg of total RNA on a 1% agarose gel to ensure the presence of intact bands of ribosomal RNAs, 18S and 28S rRNA.

Reverse transcription

RT-PCR of miRNAs was conducted according to Biggar et al. [18]. Sequences for stem-loop primers are listed in Table S1. Primers were designed based on conserved sequences from invertebrate species listed in miRBase (http://www.mirbase.org/). For reverse transcription, mature miRNAs were annealed to their respective stem loop primer using the following reaction: 5 min at 95 °C followed by 5 min at 60 °C. Reactions were then centrifuged and held on ice for 1 min. Subsequently, the following reagents were added to each reaction: 4 μL of 5× First Strand Buffer (Invitrogen), 2 μL of 100 mM dithiothreitol (Invitrogen) and 1 μL of pre-mixed dNTPs (25 μM each), 1 μL of M-MLV Reverse Transcriptase (Invitrogen), and 6 μL of DEPC-treated water. The final reverse transcription reaction was carried out as follows: 16 °C for 30 min followed by 60 cycles of 20 °C for 30 s, 42 °C for 30 s and 50 °C for 1 s followed by a final cycle of 85 °C for 5 min.

PCR amplification

PCR was carried out to amplify the miRNA-specific and 5S reverse transcription products. Forward primers for all miRNAs and 5S rRNA are listed in Table S1. A universal reverse primer (5′-CTCACAGTACGTTGGTATCCTTGTG-3′) complementary to the stem-loop sequence was used as the reverse primer in all amplifications. Amplification was carried out using miRNA-specific cDNA template, miRNA-specific forward primer and universal reverse primer with the following program: 95 °C for 10 min, followed by 30 cycles of 95 °C for 15 s and 60 °C for 1 min, with a final holding temperature of 4 °C.

The amplified products were then mixed with 2× SYBR® Green I nucleic acid gel stain (Invitrogen) and were loaded onto 2.5% agarose gels for electrophoresis. The bands from the most dilute cDNA sample that yielded a visible product were used for quantification, ensuring that PCR products did not reach amplification saturation. All PCR products were sent to BioBasic (Markham, ON, Canada) for sequencing using a shortened universal reverse primer (5′-CTCACAGTACGTTGG-3′) to increase sequence coverage.

Quantification and statistics

DNA and immunoreactive bands were visualized and analyzed using a ChemiGenius BioImaging System and GeneTools software (Syngene, MD, USA). To correct any minor variations in sample loading, immunoblot band intensity in each lane was normalized against a group of strong bands in the same lane stained by Coomassie blue. All experimental samples showed constant intensity of immunoreactive α-tubulin bands between stresses. For RT-PCR, miRNA band intensities were normalized against the intensity of corresponding 5S rRNA amplified from the same RNA sample. Mean normalized band densities ± SEM were calculated for samples from control snails and snails exposed to freezing and anoxia. Differences were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test with a significance level of $P < 0.05$.

Authors' contributions

KBS designed and coordinated the study. KKB conceived the study, carried out molecular genetic studies. SFK car-

ried out molecular genetic studies and got involved in the sequence alignment. YM carried out molecular genetic studies and the immunoassays. KKB drafted and revised the manuscript with the help of KBS All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgments

Thanks go to Janet M. Storey for editorial review of the manuscript. This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council (NSERC Grant No. 6793) of Canada. K.B. Storey holds the Canada Research Chair in Molecular Physiology, K.K. Biggar held an NSERC postgraduate fellowship, and S.F. Kornfeld and Y. Maistrovski were supported by NSERC undergraduate summer research awards.

Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gpb.2012.04.001.

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